Altered mitochondrial function in fibroblasts containing MELAS or MERRF mitochondrial DNA mutations

Andrew M. JAMES*, Yau-Huei WEI†, Cheng-Yoong PANG† and Michael P. MURPHY*‡

*Department of Biochemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand, and †Department of Biochemistry and Center for Cellular and Molecular Biology, National Yang-Ming University, Taipei, Taiwan 11221

A number of human diseases are caused by inherited mitochondrial DNA mutations. Two of these diseases, MELAS (**m**itochondrial myopathy, **e**ncephalopathy, **l**actic **a**cidosis and **s**troke-like episodes) and MERRF (**m**yoclonic **e**pilepsy and **r**agged-**r**ed **f**ibres), are commonly caused by point mutations to tRNA genes encoded by mitochondrial DNA. Here we report on how these mutations affect mitochondrial function in primary fibroblast cultures established from a MELAS patient containing an A to G mutation at nucleotide 3243 in the $tRNA^{Leu(UUR)}$ gene and a MERRF patient containing an A to G mutation at nucleotide 8344 in the $tRNA^{Lys}$ gene. Both mitochondrial membrane potential and respiration rate were significantly decreased in digitonin-permeabilized MELAS and MERRF fibroblasts respiring on glutamate/malate. A similar decrease in mitochondrial membrane potential was found in intact MELAS and MERRF fibroblasts. The mitochondrial content of these cells, estimated by stereological analysis of electron micrographs and from measurement of mitochondrial marker enzymes, was similar in control, MELAS and MERRF cells. Therefore, in cultured fibroblasts, mutation of mitochondrial tRNA genes leads to assembly of bioenergetically incompetent mitochondria, not to an alteration in their amount. However, the cell volume occupied by secondary lysosomes and residual bodies in the MELAS and MERRF cells was greater than in control cells, suggesting increased mitochondrial degradation in these cells. In addition, fibroblasts containing mitochondrial DNA mutations were 3–4-fold larger than control fibroblasts. The implications of these findings for the pathology of mitochondrial diseases are discussed.

INTRODUCTION

Human mitochondrial DNA (mtDNA) is 16569 bp in size and encodes 13 proteins comprising seven subunits of NADH: ubiquinone oxidoreductase (Complex I), one subunit of ubiquinol:cytochrome *c* oxidoreductase (Complex III), three subunits of cytochrome *c* oxidase (Complex IV) and two subunits of the F_1F_o-ATPase, along with the 22 tRNAs and two rRNAs necessary for their translation [1]. The remaining majority of mitochondrial respiratory proteins are encoded by nuclear DNA, translated in the cytoplasm, and then imported into mitochondria where they are assembled into functional complexes together with the mitochondrially encoded polypeptides [2,3]. Inherited mtDNA mutations cause a number of human diseases (for reviews see [4,5]) which primarily affect muscle and neural tissues, and their clinical severity increases with age and with the proportion of mutant mtDNA present in the target tissues [3,6].

Two mitochondrial diseases, termed MELAS (**m**itochondrial myopathy, **e**ncephalopathy, **l**actic **a**cidosis and **s**troke-like episodes), and MERRF (**m**yoclonic **e**pilepsy and **r**agged-**r**ed **f**ibres), have attracted particular interest. Both diseases are caused by point mutations in tRNA genes of mtDNA, generally an A to G transition at nucleotide 3243 in the $tRNA^{Leu(UUR)}$ gene for MELAS [7,8], and an A to G transition at nucleotide 8344 in the $tRNA^{Lys}$ gene for MERRF [9,10]. Even though the mutations are similar, these diseases are clinically distinct and it is unclear how a specific mtDNA mutation leads to each particular syndrome [11]. In both diseases there is generally a decrease in the activities of Complexes I and IV and a general deficiency in mitochondrial protein synthesis [7,9,12,13]. In MERRF there is a decrease in $tRNA^{Lys}$ aminoacylation capacity which leads to premature termination of translation, resulting in an impairment of mitochondrial protein synthesis (particularly of proteins containing a large number of lysine residues) and an accumulation of truncated mitochondrial peptides [14]. The MELAS $tRNA^{Leu(UUR)}$ mutation also impairs mitochondrial protein synthesis causing a decrease in respiratory chain complex activity, but in this case the impairment of protein synthesis does not correlate with the number of $tRNA^{Leu(UUR)}$-translated leucines in the peptide [12,13]. The cause of defective protein synthesis in MELAS cells is unclear; however, several factors may contribute, including: defective mRNA processing, an incorrectly processed transcript ('RNA 19') slowing protein synthesis by binding unproductively to ribosomes, altered kinetics of $tRNA^{Leu(UUR)}$ aminoacylation, or incorrect conjugation of amino acids to $tRNA^{Leu(UUR)}$ [13,15].

It is unclear how mtDNA mutations cause cell damage; therefore we performed experiments to determine how mtDNA mutations affect mitochondrial function in primary fibroblast cultures containing the common MELAS or MERRF mutations. These experiments included measurements of respiratory enzyme complex activity, respiration rate and mitochondrial membrane potential in intact and digitonin-permeabilized cells. In addition, we measured cell volume, the volume fraction of mitochondria, the content of secondary lysosomes and residual bodies, and the activities of mitochondrial marker enzymes. These experiments

Abbreviations used: CsA, cyclosporin A; DCIP, 2,6-dichlorophenol-indophenol; DMEM, Dulbecco's modified Eagle's medium; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; MELAS, myopathy, encephalopathy, lactic acidosis and stroke-like episodes; MERRF, myoclonic epilepsy and ragged-red fibres; mtDNA, mitochondrial DNA; TPMP, methyltriphenylphosphonium cation.

‡ To whom correspondence should be addressed.

indicate how mtDNA mutations affect mitochondrial function and suggest mechanisms whereby mtDNA mutations can decrease cell viability.

MATERIALS AND METHODS

Culture of fibroblasts

A primary skin fibroblast culture was established from a 16-year-old male Taiwanese MELAS patient whose tissues contained the 3243 point mutation at the following levels: blood cells (28.5 %), hair follicle cells (34 %), muscle biopsy (75 %) and initial fibroblast primary culture (56 %). A primary skin fibroblast culture was established from a 46-year-old male Taiwanese MERRF patient (patient II-1 of the pedigree shown in [16]) whose tissues contained the 8344 mutation at the following levels: blood (> 99 %), hair follicle cells (> 99 %), muscle biopsy (78 %) and the initial fibroblast primary culture (64 %). Three control primary skin fibroblast cultures were established, two from healthy Taiwanese adult males and one from a healthy 4-year-old New Zealand Caucasian boy; control data are the mean values from experiments on all three of these control cell cultures. Fibroblasts were grown to confluence in 150 cm^2 Petri dishes in a humidified atmosphere of 5 % CO_2/95 % air, in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (100000 units/l), streptomycin (100 mg/l) and 10 % (v/v) foetal-calf serum, supplemented with uridine (50 mg/l) and pyruvate (110 mg/l) [17,18]. Cells were harvested using 0.125 % (w/v) trypsin, washed in DMEM/10 % foetal-calf serum and resuspended in PBS. Cells were counted using an improved Neubauer haemocytometer and protein was determined by the bicinchoninic acid method using BSA as a standard [19].

Preparation of mitochondria from fibroblasts

Fibroblasts in PBS were sedimented (150 ***g*** for 5 min at 4 °C), resuspended in 8 ml of ice-cold STE buffer (250 mM sucrose, 5 mM Tris/HCl, 1 mM EGTA, pH 7.4) and disrupted with a 15 ml glass homogenizer. The nuclei were sedimented (1000 ***g*** for 5 min at 4 °C), the pellet washed and the combined supernatants were centrifuged (10000 ***g*** for 10 min at 4 °C), then the mitochondrial pellet was washed once with and then resuspended in about 1 ml of buffer containing 120 mM KCl, 10 mM Hepes/KOH, 1 mM EGTA (pH 7.4) and stored on ice. The mitochondrial fraction contained 1–2 % of the total cytosolic lactate dehydrogenase (LDH) and 50–70 % of the total cellular glutamate dehydrogenase (GDH).

Measurement of enzyme activities

All assays were conducted with an SLM Aminco DW-2000 spectrophotometer using a 1 ml sample cuvette thermostatically maintained at 30 °C. Citrate synthase [20,21], LDH [22] and GDH [23] were assayed by standard procedures. To assay respiratory enzyme complexes mitochondria were first disrupted by three cycles of freezing in solid CO_2/ethanol followed by thawing at 37 °C. Complex I activity was assayed in 10 mM Tris/HCl buffer (pH 7.4) containing 50 mM KCl, 1 mM EDTA, 500 μg/ml sonicated soybean L-α-phosphatidylcholine, 125 μM NADH, 2 mM KCN and 300 nM antimycin A_1. After addition of disrupted mitochondria, ubiquinone-1 (50 μM) was added and the rate of disappearance of NADH was monitored at 340 nm. Rotenone (20 μM) was then added to determine the background rate and the activity was calculated using $\epsilon_{340} = 6.81\ mM^{-1} \cdot cm^{-1}$, due to interference from ubiquinone-1 [24,25]. Succinate:CoQ oxidoreductase (Complex II) activity was assayed in 50 mM KH_2PO_4 buffer (pH 7.4) containing 100 μM EDTA, 0.01 % Triton X-100, 20 mM potassium succinate, 2 mM KCN, 10 μM rotenone and 300 nM antimycin A_1. After a 5 min preincubation at 30 °C to activate the enzyme, ubiquinone-2 (50 μM) and 2,6-dichlorophenol-indophenol (DCIP; 75 μM) were added and the reduction of DCIP was measured at 600 nm ($\epsilon_{600} = 21\ mM^{-1} \cdot cm^{-1}$). The background rate was measured after the addition of thenoyltrifluoroacetone (200 μM) [26]. Succinate:cytochrome *c* oxidoreductase (Complex II–III) activity was assayed in 50 mM KH_2PO_4 buffer (pH 7.4) containing 1 mM EDTA, 20 mM potassium succinate, 2 mM KCN and 10 μM rotenone. After a 5 min preincubation at 30 °C oxidized cytochrome *c* (50 μM) was added and the rate of reduction of cytochrome *c* was measured at 550 nm ($\epsilon_{550red-ox} = 21\ mM^{-1} \cdot cm^{-1}$). The background rate was measured after the addition of myxothiazol (400 nM). Complex III activity was assayed in 50 mM KH_2PO_4 buffer (pH 7.4) containing 1 mM EDTA, 50 μM oxidized cytochrome *c*, 2 mM KCN and 10 μM rotenone. After the addition of ubiquinol-2 (50 μM) the rate of reduction of cytochrome *c* was measured at 550 nm ($\epsilon_{550red-ox} = 21\ mM^{-1} \cdot cm^{-1}$) and myxothiazol (400 nM) was added to determine the background rate [27]. The activity of isolated Complex IV is dependent on ferrocytochrome *c* concentration and its activity is usually reported as the first-order rate constant for ferrocytochrome *c* oxidation [28]. In our experiments Complex IV activity was saturated at a ferrocytochrome *c* concentration of about 100 μM, in agreement with others [29], therefore we used the initial rate of oxidation of 100 μM ferrocytochrome *c* as a measure of Complex IV activity (less than 2 % of the ferrocytochrome *c* was consumed during the assay). Complex IV activity was assayed in 200 mM Tris/HCl buffer (pH 7.5) containing 10 μM EDTA, 0.3 % (v/v) Tween-80, 10 μM rotenone, 300 nM antimycin A_1 and 100 μM ferrocytochrome *c*. The absorbance of ferrocytochrome *c* at 550 nm was not linear with concentration at around 100 μM; therefore oxidation was monitored at 520 nm ($\epsilon_{520red-ox} = 6.3\ mM^{-1} \cdot cm^{-1}$). The background rate was measured after the addition of KCN (200 μM). F_1F_o-ATPase activity was assayed in 100 mM Tris/HCl buffer (pH 8.0) containing 50 mM KCl, 2 mM $MgCl_2$, 200 μM EDTA, 20 μM NADH, 1 mM phosphoenolpyruvate, 2.5 mM MgATP, 10 units/ml pyruvate kinase, 5 units/ml LDH, 2 mM KCN, 10 μM rotenone and 300 nM antimycin A_1 and the decrease in absorbance of NADH at 340 nm was measured ($\epsilon_{340} = 6.22\ mM^{-1} \cdot cm^{-1}$). Oligomycin (2.5 μM) was added to determine the background rate [24].

Measurement of respiration rate, mitochondrial membrane potential and cell volume

Fibroblasts in PBS were pelleted (150 ***g*** for 5 min at 4 °C) and resuspended in DMEM containing 10 mM Hepes/KOH (pH 7.4) and stored on ice. Immediately prior to experiments cells were pelleted by centrifugation (150 ***g*** for 2 min at 4 °C) and resuspended in the appropriate experimental medium, which had been prewarmed to 37 °C.

To measure respiration rate in digitonin-permeabilized fibroblasts, cells (about 800 μg of protein) were suspended in 250 μl of 10 mM Hepes-KOH (pH 7.4), 120 mM KCl, 1 mM EGTA containing 100 μg of digitonin/ml and either 10 mM succinate and 50 μM rotenone, or 5 mM glutamate and 5 mM malate, in a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) thermostatically maintained at 37 °C. Data were acquired and analysed using a MacLab system and the oxygen electrode was calibrated with air-saturated water assuming 406 nmol of O atoms/ml at 37 °C [30]. Oxygen consumption was measured over 10–15 min with sequential additions

of oligomycin (25 μM), carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 200 nM) and myxothiazol (800 nM) to measure coupled, uncoupled, and non-mitochondrial oxygen consumption, respectively. Non-mitochondrial oxygen consumption, typically 0.5–1.0 nmol/min per mg of protein, was subtracted from all measurements to give mitochondrial respiration rates.

To measure mitochondrial membrane potential in digitonin-permeabilized fibroblasts, cells (about 160 μg of protein) were incubated in 250 μl of 10 mM Hepes-KOH (pH 7.4), 120 mM KCl, 1 mM EGTA containing 1 μM methyltriphenylphosphonium (TPMP) bromide, 100 nCi/ml [^{3}H]TPMP, 20 μg/ml digitonin and either 10 mM succinate and 50 μM rotenone, or 5 mM glutamate and 5 mM malate. After a 10 min incubation at 37 °C the cells were pelleted by centrifugation (10000 ***g*** for 20 s) and 50 μl of the supernatant was transferred to a scintillation vial. The remaining medium was aspirated and the pellet resuspended in 40 μl of 20 % (v/v) Triton X-100 and transferred to a scintillation vial. Scintillant (3.75 ml of OptiPhase 'Hisafe' 2) was added and the radioactivity was quantified using an LKB Wallac RackBeta liquid scintillation counter with appropriate quench corrections. The mitochondrial membrane potential-independent TPMP accumulation was determined by parallel incubations supplemented with FCCP (400 nM) and was subtracted from the accumulation by coupled mitochondria to give the membrane potential-dependent TPMP accumulation. The uptake of TPMP into the permeabilized cells was expressed as an accumulation ratio in the units of [(TPMP/mg of protein)/(TPMP/μl of supernatant)].

To measure mitochondrial membrane potential in intact fibroblasts, cells (about 160 μg of protein) were suspended in 250 μl of DMEM containing 10 mM Hepes/NaOH (pH 7.4), 1 μM TPMP, 500 nM sodium tetraphenylboron and 100 nCi/ml [^{3}H]TPMP and incubated for 80 min at 37 °C. After incubation the cells were sedimented (10000 ***g*** for 20 s) and prepared for scintillation counting as described above. To determine the TPMP binding to de-energized cells, parallel experiments were conducted in the above medium supplemented with 12.5 μM oligomycin, 20 μM FCCP, 1 μM myxothiazol, 100 nM valinomycin and 1 mM ouabain. The energization-dependent TPMP uptake, due to the mitochondrial and plasma membrane potentials, was expressed as an accumulation ratio in units of [(TPMP/mg of protein)/(TPMP/μl of supernatant)].

Cell volume was determined by incubating fibroblasts (640 μg of protein) in 250 μl of DMEM containing 10 mM Hepes/NaOH (pH 7.4), 100 μg/ml inulin and 1 μCi/ml ^3H_2O at 37 °C for 10 min. Inulin (200 nCi of ^{14}C/ml) was added and the incubation was then mixed, centrifuged (10000 ***g*** for 20 s) and prepared for scintillation counting as described above. The amounts of ^{14}C and ^{3}H in the supernatant and pellet samples were determined by dual-isotope scintillation counting, using appropriate crossover corrections, and the cell volume was taken as the difference between the ^3H_2O- and [^{14}C]inulin-accessible spaces in the cell pellet.

Transmission electron microscopy

Fibroblasts were grown to confluence on Melinex film, fixed with 2.5 % (v/v) glutaraldehyde in 0.09 M cacodylate buffer (pH 7.2) containing 3 mM $CaCl_2$, post-fixed in OsO_4, washed in water, stained with uranyl acetate, dehydrated in ethanol, and embedded in Araldite. Electron micrographs of thin sections were obtained using a Phillips 410 transmission electron microscope. The volume fractions of cytoplasmic components were measured by a stereological approach by overlaying high-contrast 20 cm × 25 cm photographs (× 19000 enlargement; 16 for each determination) with a 15 mm square grid and counting points of intersection; each determination was based on a total of 1600–2200 points of intersection with the cytoplasm.

DNA extraction and analysis

Total cellular DNA was extracted from 1×10^6 cells by standard procedures, dried by centrifugation under vacuum and stored at −80 °C under argon. Analysis of the proportion of mutant mtDNA in the fibroblasts was carried out according to the methods developed in the Taiwan laboratory [16,31].

Materials

DMEM and heat-inactivated foetal-calf serum were purchased from Life Technologies Inc., tissue-culture plasticware was from Nunc, trypsin was from Difco Laboratories (Michigan, U.S.A.). Melinex film was from Agar Scientific Ltd. (Stansted, Essex, U.K.). Ubiquinone-2 was a gift from Eisai Pharmaceuticals (Tokyo, Japan) and ubiquinone-1 was a gift from Hoffman–La Roche AG (Basel, Switzerland). Cyclosporin A (CsA) was a gift from Sandoz Pharma AG (Basel, Switzerland). [^{14}C]Inulin (16.5 mCi/g) and [^{3}H]TPMP (39.7 Ci/mol) were supplied by NEN Ltd. and ^3H_2O (1 mCi/ml) was from Amersham. All other chemicals were from Sigma or Boehringer Mannheim.

RESULTS AND DISCUSSION

Proportion of mutant mtDNA in MELAS and MERRF cells

The proportion of mutant mtDNA in the MELAS cells was 46 % which is similar to that present in the initial primary fibroblast culture established from the patient (56 %). For the MERRF cells the proportion of mutant mtDNA ranged from 59 % to 65 % over these experiments, which is similar to the proportion of mutant mtDNA in the initial fibroblast culture established from the patient (64 %).

Activity of respiratory chain complexes and mitochondrial enzymes

The respiratory enzyme activities in the mitochondrial fractions from the MELAS, MERRF and control cells were determined as described in the Materials and methods section (Table 1). In addition, the activities of the respiratory enzymes in the MELAS and MERRF cells, normalized against citrate synthase activity, are also expressed as a percentage of the normalized activity in control cells. This procedure eliminates any changes in activity due to a general depletion of mitochondrial enzymes or to differences in mitochondrial yield.

These experiments established that the activity of Complex I was decreased in both types of mutant cells, with very little activity detectable in the MERRF fibroblasts. Complex IV activity was also substantially reduced in MERRF cells, but not in the MELAS cells. The activity of Complex III was not affected significantly in either type of mutant cell, while the activity of Complex II was increased in MELAS fibroblasts, leading to elevated Complex II–III activity in MELAS cells.

Properties of MELAS and MERRF fibroblasts

The MERRF fibroblasts acidified the culture medium more rapidly than control cells, suggesting increased anaerobic glycolysis. To test this we measured cytoplasmic LDH activity and compared this with the citrate synthase activity to estimate the

Table 1 Mitochondrial enzyme activities in MELAS, MERRF and control fibroblasts

The activities of mitochondrial enzymes were measured as described in the Materials and methods section. Activities are expressed as nmol/min per mg of mitochondrial protein and also as a percentage of activities in control cell extracts, normalized against citrate synthase activity. The data are the means ± S.E.M of results from the number of experiments indicated in parentheses for the MELAS and MERRF cells. For the control cells the data are the means ± S.E.M for experiments on three different control cells. Statistical significance was determined relative to values for control cells by Student's two-tailed *t*-test. *, $P < 0.05$; ***, $P < 0.001$.

Enzyme	Control	MELAS	MERRF
Citrate synthase	315 ± 21	291 ± 6 (6)	180 ± 30 (3)
GDH	431 ± 63	547 ± 63 (4) 115%	211 ± 30 (3) 74%
Complex I	27.1 ± 2.8	19.0 ± 1.5 (3) 76%*	3.5 ± 1.6 (3) 22%***
Complex II	70.2 ± 16.8	120.9 ± 4.7 (3) 189%*	43.5 ± 5.9 (3) 109%
Complex II–III	74.5 ± 26.7	95.6 ± 10.1 (3) 141%	30.8 ± 4.8 (3) 73%
Complex III	470 ± 50	405 ± 25 (3) 94%	196 ± 23 (3) 72%
Complex IV	57.0 ± 30.3	54.7 ± 3.1 (3) 108%	12.8 ± 1.5 (3) 41%
ATPase	163.7 ± 24.0	134.4 ± 23.7 (3) 88%	60.0 ± 7.2 (3) 63%*

Table 2 LDH/citrate synthase ratio in MELAS, MERRF and control fibroblasts

LDH activity was measured as nmol/min per mg of cell protein in cell homogenates as described in the Materials and methods section. Citrate synthase activity was measured in isolated mitochondria and converted into nmol/min per mg of cell protein from the known yield of mitochondrial protein. The ratios of these two values are shown in the Table. These data are the means ± S.E.M. of results from at least three separate determinations, for the MELAS and MERRF cells, and the means ± S.E.M. of results from experiments on the three different control cell cultures. Statistical significance was calculated relative to control values by Student's two-tailed *t* test. *, $P < 0.05$.

Cell type	LDH/citrate synthase ratio
Control	40.8 ± 8.7
MELAS	73.5 ± 13.7
MERRF	89.8 ± 12.5*

relative oxidative and anaerobic capacities of these cells. We found an increase in the LDH/citrate synthase ratio in both the MELAS and MERRF cells relative to control cells (Table 2).

Table 4 Volume fraction of mitochondria, secondary lysosomes and residual bodies in MELAS, MERRF and control fibroblasts

The volume fractions of the organelles were determined by stereological analysis of electron micrographs of cell monolayers as described in the Materials and methods section. Data are the means ± range for determinations on two separate samples from MELAS and MERRF cells and from a control cell culture.

	Cytoplasmic volume fraction (%)		
Cell component	Control	MELAS	MERRF
Mitochondria	2.4 ± 0.5	1.7 ± 0.2	2.1 ± 0.1
Secondary lysosomes	2.7 ± 0.9	2.7 ± 0.2	12.4 ± 2.6
Residual bodies	5.3 ± 2.1	9.7 ± 2.3	2.3 ± 1.6

This suggests that, as expected, the defect in mitochondrial function leads to an up-regulation of the anaerobic glycolytic pathway.

Light microscopy of cell monolayers suggested that the MELAS and MERRF cells were larger than control cells and the diameters of detached, spherical MELAS and MERRF cells were about 1.5–2-fold larger than control cells, by both light and transmission electron microscopy (results not shown). Supporting these observations MERRF and MELAS fibroblasts contained 3–4-fold more protein per cell than control fibroblasts (Table 3). Direct measurement of cell volume by the exclusion of the cell-impermeant macromolecule [^{14}C]inulin relative to the cell-permeant $^{3}H_2O$ (Table 3) confirmed that the MELAS and MERRF cells were 3–4-fold larger than control fibroblasts. The cell volume per mg of protein was similar (4.7–6 μl/mg of protein) for all three cell types (Table 3) and was similar to an earlier report of 4.2 μl/mg of protein [32]. In addition, the proportion of cell protein that sedimented in the mitochondrial fraction (13.9–16.4%; after correction for loss of the mitochondrial marker enzyme GDH during isolation) was similar for MELAS, MERRF and control cells (Table 3) and was similar to a value of 12% reported by others [32]. These data suggest that the substantial change in size which occurred in the MELAS and MERRF cells did not grossly affect protein levels in our cell or mitochondrial preparations and that comparison between cell lines is valid.

To determine whether mutant mtDNA affected the proportion or morphology of mitochondria, transmission electron micrographs of cells were prepared. Stereological analysis of these electron micrographs showed no major differences in the volume fraction of the cytoplasm occupied by mitochondria in the MELAS or MERRF cells as compared with one of the control cell cultures (Table 4). The volume fraction of the cytoplasm

Table 3 Size and composition of the MELAS, MERRF and control fibroblasts

Cell volume was estimated as described in the Materials and methods section. The mitochondrial protein fraction is the percentage of cellular protein recovered in the mitochondrial fraction after correction for the yield of GDH. These data are the means ± S.E.M. of results from the number of experiments indicated in parentheses for the MELAS and MERRF cells. For the control cells, data are the means ± S.E.M. of results from experiments on the three different control cell cultures. Statistical significance was calculated relative to values for control cells by Student's two-tailed *t*-test. **, $P < 0.01$; ***, $P < 0.001$.

Measurement	Control	MELAS	MERRF
Protein per cell (pg)	599 ± 119	2374 ± 125 (11)***	1968 ± 141 (15)***
Volume per cell (pl)	2.8 ± 0.7	10.3 ± 1.3 (3)**	11.1 ± 1.4 (3)**
Volume (μl/mg of protein)	4.7 ± 0.4	4.8 ± 0.8 (3)	6.0 ± 0.3 (3)
Mitochondrial protein fraction (%)	15.5 ± 0.5	13.9 ± 0.8 (4)	16.4 ± 1.5 (3)

Table 5 Respiration rate in digitonin-permeabilized MELAS, MERRF and control fibroblasts

Mitochondrial oxygen consumption was measured in digitonin-permeabilized fibroblasts as described in the Materials and methods section using succinate or glutamate/malate. Uncoupled and coupled respiration rates are the means ± S.E.M. of three independent experiments for the MELAS and MERRF cells and are the means ± S.E.M. for experiments on the three different control cell cultures. Statistical significance was calculated relative to control values using Student's two-tailed *t*-test *, $P < 0.05$. The ratio of uncoupled to coupled respiration rate is also shown.

Cell type	Respiratory substrate	Coupled respiration rate (nmol O atoms/min per mg of cell protein)	Uncoupled respiration rate (nmol O atoms/min per mg of cell protein)	Uncoupled/coupled ratio
Control	Succinate	3.4 ± 1.3	11.2 ± 3.6	3.5 ± 0.3
MELAS	Succinate	3.3 ± 0.99	8.7 ± 0.07	3.0 ± 0.71
MERRF	Succinate	1.6 ± 0.18	4.5 ± 0.3	2.8 ± 0.41
Control	Glutamate/malate	1.8 ± 0.9	6.9 ± 1.8	4.8 ± 1.2
MELAS	Glutamate/malate	1.1 ± 0.43	1.4 ± 0.5*	1.4 ± 0.4
MERRF	Glutamate/malate	1.2 ± 0.22	1.2 ± 0.19*	1.3 ± 0.36*

Table 6 Mitochondrial TPMP accumulation by digitonin-permeabilized MELAS, MERRF and control fibroblasts

Mitochondrial TPMP accumulation was measured in digitonin-permeabilized cells as described in the Materials and methods section. Glutamate/malate or succinate were used to energize the mitochondria. These data are the means ± S.E.M. of results obtained from three experiments for the MELAS and MERRF cells. For the control cells these data are the means ± S.E.M. for experiments on the three different control cell cultures. Statistical significance is calculated relative to control values using Student's two-tailed *t*-test. *, $P < 0.05$; **, $P < 0.01$.

	Accumulation ratio (TPMP/mg of protein)/(TPMP/μl)		
Respiratory substrate	Control	MELAS	MERRF
Succinate	51.5 ± 9.9	14.3 ± 5.7*	13.5 ± 2.9*
Glutamate/malate	54.2 ± 5.7	6.4 ± 4.4**	12.1 ± 4.9**

occupied by the mitochondria (1.7–2.4 %) was similar to the volume (3.3–4 %) obtained by others [33]. While the morphology of these mitochondria was grossly normal, we noted an increase in the volume fraction of secondary lysosomes, and/or residual bodies, in MELAS and MERRF cells relative to the control cell culture (Table 4). One pathway of mitochondrial degradation is through fusion of mitochondria with primary lysosomes to form secondary lysosomes. However, not all components are effectively degraded within the secondary lysosomes, leading to the formation of residual bodies, which are thought to be undigested remnants of damaged mitochondria, and other cellular components [34]. These data suggest that in the MELAS and MERRF cells there may be increased turnover and degradation of mitochondria relative to control cells.

Bioenergetics of digitonin-permeabilized MELAS and MERRF fibroblasts

Digitonin-permeabilized cells are an excellent surrogate for isolated mitochondria because digitonin removes the permeability barrier of the plasma membrane, exposing the mitochondria to analysis. In control experiments (results not shown) we determined the optimal amount of digitonin (30 μg/mg of cell protein) required for plasma membrane permeabilization without damaging mitochondria, by measuring the respiration rate and mitochondrial membrane potential of cells in the presence of succinate.

Mitochondria in the permeabilized cells were energized with succinate, which bypasses Complex I (donating electrons to the respiratory chain at Complex II), or a mixture of glutamate and malate, which donates electrons to the respiratory chain at Complex I via NADH. We found little change in the succinate-supported respiration rate of coupled mitochondria in MERRF or MELAS fibroblasts (Table 5). When glutamate/malate was used as a respiratory substrate (Table 5) there was a substantial decrease in MERRF cells which was also present for MELAS cells. Respiration rates were also measured in permeabilized fibroblasts treated with the uncoupler FCCP, which gives the maximum possible activity of the respiratory chain [35]. Respiration rate was decreased by about 23 % and 60 % using succinate and 81 % and 82 % using glutamate/malate in MELAS and MERRF cells, respectively. Therefore, the defects in the respiratory enzyme complexes dramatically affected the kinetics of the respiratory chain when glutamate/malate was used as a respiratory substrate. However, when succinate was used as a respiratory substrate the effects were far smaller. The degree of mitochondrial coupling, measured as the ratio of the uncoupled to coupled respiration rates, was similar for all three cell types with succinate as the respiratory substrate but was decreased in both MELAS and MERRF cells relative to controls when glutamate/malate was the substrate (Table 5).

To determine whether the decrease in respiration rate in MELAS and MERRF cells decreased mitochondrial energization, we measured the mitochondrial membrane potential in digitonin-permeabilized fibroblasts. The lipophilic cation TPMP was used to measure the mitochondrial membrane potential because its accumulation is related to the membrane potential by the Nernst equation. However, the TPMP accumulation ratio must be corrected for mitochondrial volume and energization-dependent membrane binding of TPMP to calculate the membrane potential. As these corrections may distort comparisons between cell types, we have calculated the uptake of TPMP by MELAS and MERRF cells as an accumulation ratio (Table 6). The mitochondrial accumulation of TPMP was substantially greater in permeabilized control fibroblasts than in MELAS or MERRF cells when either succinate or glutamate/malate was used as respiratory substrate (Table 6). This decrease in TPMP accumulation is due to a decrease in

Table 7 Mitochondrial TPMP accumulation by intact MELAS, MERRF and control fibroblasts

The accumulation of TPMP into intact fibroblasts was measured as described in the Materials and methods section in the presence or absence of 500 nM CsA. The data are the means ± S.E.M. of the results obtained from three independent experiments for the MELAS and MERRF cells. Control data are the means ± S.E.M. for experiments on the three different control cell cultures. Statistical significance was calculated relative to control values using Student's two-tailed *t*-test. **, $P < 0.01$.

	Accumulation ratio (TPMP/mg of protein)/(TPMP/μl)		
	Control	MELAS	MERRF
−CsA	169.7 ± 17.4	40.3 ± 5.2**	61.2 ± 11.4**
+CsA	163.9 ± 13.6	29.2 ± 2.1	50.2 ± 10.7

mitochondrial membrane potential, not to an alteration in their amount, because MELAS, MERRF and control cells contain a similar proportion of mitochondria (Tables 3 and 4).

To estimate the mitochondrial membrane potential in the digitonin-permeabilized cells we used the cell volume (Table 3) and the mitochondrial volume fraction (Table 4) of the MELAS and MERRF cells and one of the control cell cultures to calculate the mitochondrial TPMP concentration, assuming that 60 % of the intramitochondrial TPMP was membrane-bound [36]. From the Nernst equation, we obtained values of 150 mV, 118 mV and 100 mV for control, MELAS and MERRF cells, respectively, on energization with succinate. As this calculation depends on a number of variables there is some uncertainty in the absolute value of the membrane potential; however, as differences in membrane potential are less dependent on these variables, it is likely that the mitochondrial membrane potential in MELAS and MERRF cells is 30–50 mV lower than in control cells.

Mitochondrial membrane potential in intact MELAS and MERRF fibroblasts

We also measured the accumulation of TPMP by intact MELAS, MERRF and control fibroblasts (Table 7). The accumulation of TPMP by control fibroblasts was substantially greater than that by MELAS or MERRF fibroblasts (Table 7), suggesting a major decrease in mitochondrial membrane potential, as was seen for the digitonin-permeabilized cells. The decrease in the accumulation of TPMP (Table 7) is not due to alterations in the amount of mitochondria, because the mitochondrial content is similar for the three cell types (Tables 3 and 4). In addition, it is unlikely that the alterations in the accumulation of TPMP are caused by changes in the plasma membrane potential because the plasma membrane potential of MELAS fibroblasts is very similar to that of control cells [37].

Our TPMP uptake data are presented as accumulation ratios to eliminate the assumptions necessary in calculating the mitochondrial membrane potential. To estimate the mitochondrial membrane potential from these data for the MELAS and MERRF cells and one of the control cell lines we calculated the cytoplasmic concentration of TPMP from the plasma membrane potential (−48.5 mV) [37], using the Nernst equation. From the cytoplasmic concentration of TPMP and the cell volume (Table 3) the cytoplasmic proportion of TPMP taken up by the cell was calculated. This was subtracted from the total amount of cellular TPMP to give the amount of TPMP accumulated by the mitochondria. From the cell volume (Table 3) and the mitochondrial volume fraction of the cell (Table 4) we calculated the TPMP concentration in the mitochondrial matrix, assuming that 60 % of the intramitochondrial TPMP was membrane-bound [36]. Finally, we calculated the mitochondrial membrane potential by inserting the values for the cytoplasmic and mitochondrial TPMP concentrations into the Nernst equation. These calculations gave mitochondrial membrane potentials of 124 mV, 69 mV and 68 mV for control, MELAS and MERRF cells, respectively. For comparison, others have reported values of 150 mV in hepatocytes and 127 mV in thymocytes [38]. Substantially larger mitochondrial membrane potentials of 167–175 mV have been reported in normal fibroblasts [33]; however, the difference between our data and this earlier report is due to the different correction procedures used and not to any difference in the experimental measurements. One possible contribution to the discrepancy may be that the intramitochondrial binding of the tetraphenylphosphonium cation was not taken into account in this earlier work. Because of the many correction factors, the absolute mitochondrial membrane potential in our cells is uncertain. However, it is likely that the mitochondrial membrane potential is about 50 mV lower in MELAS and MERRF cells than in control cells.

In the mitochondrial inner membrane there is a non-specific CsA-sensitive pore which opens under calcium loading or oxidative stress, depolarizing the mitochondria [39]. The role of this mitochondrial permeability transition is unclear but it may contribute to cell damage under pathological conditions. As elevated cytosolic calcium has been shown in MELAS cells [37] we determined whether this CsA-sensitive permeability transition was induced in MELAS or MERRF fibroblasts. To do this we measured mitochondrial TPMP accumulation in control, MELAS and MERRF fibroblasts in the presence or absence of CsA (Table 7). If the mitochondrial permeability transition contributes to the decrease in TPMP accumulation seen in MELAS or MERRF cells, then incubation with CsA would reverse this depolarization. However, CsA did not increase TPMP accumulation (Table 7), suggesting that the mitochondrial permeability transition does not contribute to the decrease in mitochondrial membrane potential in MELAS or MERRF cells.

Conclusions

The MELAS or MERRF point mutations in mitochondrial tRNA genes did not lead to an alteration in the amount of grossly normal mitochondria, but in both MELAS and MERRF cells there was an increase in the number of secondary lysosomes and residual bodies relative to control cells. A plausible explanation is that in MELAS or MERRF cells the defective mitochondria are rapidly degraded and we are currently testing this hypothesis.

The impaired respiratory enzyme activity in MELAS and MERRF leads to decreases in mitochondrial respiration rate and membrane potential in digitonin-permeabilized cells. There was a similar decrease in mitochondrial membrane potential of about 50 mV in intact MELAS or MERRF cells relative to controls. This supports and extends an earlier observation that MELAS cells have a decreased mitochondrial membrane potential [37], which was based on semi-quantitative measurement of mitochondrial polarization by J-aggregate formation of the fluorescent dye JC-1. Therefore, in both MELAS and MERRF fibroblasts even a relatively low proportion of mutant mtDNA (46–65 %) significantly decreases the mitochondrial membrane potential. The decrease in membrane potential seen for both MELAS and MERRF cells respiring on glutamate/malate may be largely ascribed to a decrease in electron movement though the respiratory chain, as the respiration rate supported by these

substrates is substantially decreased. However, when succinate is used as a respiratory substrate there is just as substantial a decrease in membrane potential for MELAS cells, even though the succinate-supported respiration rate is similar to that of control cells; consistently with this, the only respiratory defect in these MELAS cells is in Complex I, which would not affect succinate-supported respiration. This suggests that the decrease in membrane potential is not simply due to a decrease in electron movement through the respiratory chain. Currently we are investigating how relatively low proportions of mtDNA mutations which do not diminish respiration rate lead to a major decrease in mitochondrial membrane potential.

This decrease in membrane potential will have a number of deleterious consequences for the cell. A major effect will be on ATP synthesis, and in muscle and neuronal cells glycolysis will be incapable of providing sufficient ATP for normal cell function. Decreasing the mitochondrial membrane potential will also affect cellular calcium homoeostasis [39]. It has been shown that the decrease in mitochondrial membrane potential in MELAS cells led to elevated resting intracellular calcium and impaired the cell's ability to handle excess calcium influx [37]. As disruption to calcium homoeostasis is an important factor in cell death this aspect of mitochondrial dysfunction in MELAS and MERRF cells may contribute significantly to cell death in patients with these syndromes. Intriguingly, the volume of MELAS and MERRF fibroblasts was substantially larger than control cells. The reason for this is unclear but suggests mitochondrial dysfunction has a number of unexpected consequences for cell function.

This work was supported by grants to M. P. M. from the Health Research Council of New Zealand, the Anderson and Telford Charitable Trust and the NZ Ministry of Research, Science and Technology Bilateral Research Activities Programme, and was partly supported by a grant (DOH 84-HR-219) to Y.-H. W. from the Department of Health, Executive Yuan, Republic of China. Y.-H. W. is grateful to the University of Otago for the award of a William Evans Fellowship. We thank Alan Mitchell, Richard Lander and Deborah Macleod for expert assistance with the electron micrographs, Associate Professor David Rayns and Dr. Peter Hurst for helpful discussions and Drs. Michael J. Hubbard and Serena J. Hyslop for their comments on the manuscript. We thank Judy Norrish for assistance with cell cultures.

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Received 30 November 1995/2 May 1996; accepted 7 May 1996